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BK Polyomavirus Replication in Renal Tubular Epithelial Cells Is Inhibited by Sirolimus, but Activated by Tacrolimus Through a Pathway Involving FKBP-12

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BK polyomavirus (BKPyV) replication causes nephropathy and premature kidney transplant failure. Insufficient BKPyV-specific T cell control is regarded as a key mechanism, but direct effects of immunosuppressive drugs on BKPyV replication might play an additional role. We compared the effects of mammalian target of rapamycin (mTOR)- and calcineurin-inhibitors on BKPyV replication in primary human renal tubular epithelial cells. Sirolimus impaired BKPyV replication with a 90% inhibitory concentration of 4 ng/mL by interfering with mTOR–SP6-kinase activation. Sirolimus inhibition was rapid and effective up to 24 h postinfection during viral early gene expression, but not thereafter, during viral late gene expression. The mTORC-1 kinase inhibitor torin-1 showed a similar inhibition profile, supporting the notion that early steps of BKPyV replication depend on mTOR activity. Cyclosporine A also inhibited BKPyV replication, while tacrolimus activated BKPyV replication and reversed sirolimus inhibition. FK binding protein 12kda (FKBP-12) siRNA knockdown abrogated sirolimus inhibition and increased BKPyV replication similar to adding tacrolimus. Thus, sirolimus and tacrolimus exert opposite effects on BKPyV replication in renal tubular epithelial cells by a mechanism involving FKBP-12 as common target. Immunosuppressive drugs may therefore contribute directly to the risk of BKPyV replication and nephropathy besides suppressing T cell functions. The data provide rationales for clinical trials aiming at reducing the risk of BKPyV replication and disease in kidney transplantation.

Abbreviations: BKPyV, BK polyomavirus; CsA, cyclosporine A; EVGR, early viral gene region; FKBP-12, FK binding protein 12kda; KT, kidney transplantation; LTag, large T-antigen; LVGR, late viral gene region; sTag, small T-antigen; SIR, sirolimus; TAC, tacrolimus; VP1, viral capsid protein 1

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Introduction

In the past decade, BK polyomavirus (BKPyV)–associated nephropathy has surfaced as a significant cause of premature kidney transplantation (KT) failure (1–4). BKPyV-associated nephropathy is preceded by high-level BKPyV viruria and viremia (5). This observation is translated clinically by screening KT patients for significant BKPyV replication and guiding preemptive reduction of maintenance immunosuppression (6–8). Although never formally tested in randomized clinical trials, this strategy is successful according to dedicated prospective studies reporting favorable virological, immunological, and functional outcomes (9–14). Failure to regain immune control over BKPyV replication is linked to emergence of more pathogenic viral variants, histological progression, and loss of allografts (15,16). This condition has been associated with a variety of risk factors (e.g. male recipient, older recipient age, higher number of HLA mismatches, acute rejection episodes, higher steroid exposure, ureteric stents, as well as transplanting organs from donors with high BKPyV antibody titers into recipients with low or undetectable antibody titers) (5,9,13,17–25).

The direct contribution of immunosuppressive drugs has been a matter of debate. BKPyV-associated nephropathy has been sporadically reported prior to the cyclosporine-A (CsA) era (26,27), but most cases have emerged after widespread clinical use of tacrolimus (TAC) and TAC-mycophenolate (28–32). Data from large registries of 35 000 and more KT patients report a significantly increased risk of BKPyV for TAC- compared to CsA-based regimens, while a reduced risk of BKPyV was seen for mammalian target of rapamycin (mTOR) inhibitor-containing therapies (21,22). Since overall rates of acute rejection in the first year after KT have declined in the past decade, it seems

likely that more intense immunosuppression plays a key role, and that CsA-based or mTOR inhibitor-based therapies may simply be less potent. However, recent data from two large prospective randomized trials demonstrated a reduced risk of BKPyV events in CsA- and mTOR inhibitor arms compared to the standard TAC- or mycophenolate-based arm in *de novo* KT (24,33,34). In these randomized prospective trials, noninferiority was observed regarding biopsy-proven acute rejection, or related composite end points after 6 or 12 months posttransplant (33,34). Thus, while the overall immunologic potency appears to be a plausible key component increasing the risk of BKPyV replication (35,36), differences between immunosuppressive drugs might play an additional role (37). We therefore examined the direct virological effects of the mTOR inhibitor sirolimus (SIR) and of the calcineurin inhibitors TAC and CsA on BKPyV replication in a well-characterized model of primary human proximal renal tubular epithelial cells (RPTECs), the primary target of BKPyV in the renal allograft (38,39).

Materials and Methods

Cell culture, infection with BKPyV, and treatment with drugs

Primary RPTECs were purchased from different providers (ATCC, Manassas, VA; lot 58488852, 13-month-old donor; ScienCell, Carlsbad, CA; lot 5111, 3-month-old donor; Lonza, Basel, Switzerland). RPTECs were cultured in epithelial cell medium (EpiCM; ScienCell), supplemented with epithelial cell growth supplement (EpiCGS; ScienCell) and 2% fetal bovine serum (FBS; ScienCell). RPTECs were seeded and left to adhere overnight at 37°C followed by medium change and further expansion as required. For cell starvation, RPTECs were seeded and cultured in epithelial cell growth medium without supplements for 36 to 48 h. Purified BKPyV-Dunlop was prepared as previously described (39). BKPyV-viral capsid protein 1(VP1)–derived virus-like particles (VLP) were prepared as described (40,41) and added to RPTECs. Medium with virus or VLP preparation was removed, and cells were washed three times and fresh medium was added without or with drugs indicated in the figures in the results: SIR (rapamycin; dissolved in dimethylsulfoxide [DMSO]; Sigma-Aldrich, St. Louis, MO), TAC (FK506; dissolved in DMSO; Sigma-Aldrich), cyclosporin A (FK506; dissolved in DMSO; Sigma-Aldrich), Torin1 (dissolved in DMSO; Sigma-Aldrich).

FKBP-12 siRNA knockdown

Cells were seeded in a T25 flask and left to adhere overnight at 37°C. Human FK binding protein 12kda (FKBP-12) siRNA (Santa Cruz, Dallas, TX) was used, while control siRNA-A (Santa Cruz) was used to control for off-target effects. The siRNAs were delivered by Lipofectamine RNAiMAX (Life Technologies, Carlsbad, CA), and Opti-MEM+GlutMAX (Life Technologies) was used, as described by the manufacturer. After 5 h, medium was replaced and the cells were cultured for 1 or 2 days prior to further experimenting.

Immunofluorescence staining, microscopy, and image analysis

The cells on coverslips were fixed at 72 h postinfection (hpi) with 4% formaldehyde (PFA) (10% PFA, Polysciences, Eppelheim, Germany) diluted in phosphate-buffered saline (PBS) (with Ca^{2+} Mg^{2+}) for 10 min and permeabilized with 0.2% Triton X-100 (10%, Sigma-Aldrich) for 10 min at room temperature (RT). Fixed cells were blocked with blocking buffer containing milk powder (Coop) and PBS (with Ca^{2+} Mg^{2+}) for 15 min (37°C). The primary and secondary antibodies were diluted in blocking buffer and incubated at RT for 50 min each. The primary antibodies were monoclonal mouse anti-VP1 (1:300; 10309-5E6; Abnova, Taipei City, Taiwan), polyclonal rabbit anti-agnoprotein (1:800) (42), and monoclonal mouse anti-simian virus 40 (SV40) large T-antigen (LTag) (1:50; DP02; Merck, Darmstadt, Germany). The secondary antibodies were anti-mouse IgG1–Alexa Fluor 647 (1:800; A-21240; Life Technologies), anti-rabbit IgG–Alexa Fluor 488 (1:1000; A-21441; Life Technologies), IgG2a–Alexa Fluor 568 (1:300; A-21134; Life Technologies), and Hoechst 33342 dye (0.5 μg/mL; H21492; Life Technologies). After labeling with antibodies, the sample was mounted on microscope slides with ProLong Gold antifade reagent (4′,6-diamidino-2-phenylindole; P36935; Life Technologies). The visualization of the samples was made with the epifluorescence microscope (TE200; Nikon, Tokio, Japan) and the digital camera (Hamamatsu, Hamamatsu, Japan). The images were taken at 10× objective magnification and then processed by ImageJ (U.S. National Institutes of Health, Bethesda, MD).

Intracellular and extracellular DNA extraction and BKPyV load analysis

At 72 hpi, 100 μL of extracellular supernatant was collected. DNA was extracted using a Corbett X-tractor Gene and Corbett VX reagents (Qiagen, Hombrechtikon, Switzerland). BKPyV loads were quantified by real-time polymerase chain reaction as described (43). Intracellular DNA was extracted after trypsinizing (trypsin/ethylenediaminetetraacetic acid [EDTA]; Lonza) adherent cells, resuspension, and washing in PBS (without Ca^{2+} Mg^{2+}) and 2% fetal calf serum (Biochrom AG, Berlin, Germany), and centrifugation at 1250 g for 10 min at 4°C. The cell pellet was resuspended in 200 μL of ice-cold PBS (without Ca^{2+} Mg^{2+}), and DNA was extracted using the QIAamp DNA Blood Mini Kit and Qiagen Proteinase K (Qiagen, Venlo, Netherlands).

Cell lysate

Medium was removed from the cells, washed with PBS (without Ca^{2+} Mg^{2+}). Lysis buffer (0.05% NP-40 [Nonidet P40; Roche, Basel, Switzerland], 50 mM Tris-HCl, 150 mM NaCl, 1 mM of DTT [dithiothreitol; Sigma-Aldrich], 5 mM EDTA [Sigma-Aldrich], with protease inhibitor cocktail tablet [Roche, Basel, Switzerland]) was then applied and incubated at 4°C for 15 min. The cell lysate was scraped off with a cell scraper (Sarstedt, Nuümbrecht, Germany), vortexed, and incubated on ice for 10 min. Thereafter, the lysates were centrifuged at 21 130 g for 20 min (4°C). The supernatant was recovered, protein concentration was measured with BioPhotometer (Eppendorf, Hamburg, Germany), and 4× Tricine Sample Buffer (Sigma-Aldrich) was added and could be stored at −20°C.

Western blotting

The samples were loaded onto 7.5%, 10%, 15%, 4–20% gradient Tris-Tricine gels and were run in a gel electrophoresis apparatus (Biorad, Hercules, CA) with a voltage of 0.03A/gel for 2 h. The gel was blotted onto the Immobilon-FL 0.45 μm membrane (Millipore, Darmstadt, Germany), later blocked with Odyssey Blocking Buffer (LI-COR, Lincoln, NE) and TBST (1xTBS with 0.5% Tween-20; Sigma-Aldrich). The primary antibodies were diluted in the blocking buffer and incubated with the membrane overnight (4°C). The primary antibodies used were as follows: monoclonal mouse anti-α-tubulin (1:1000; Life Technologies), monoclonal mouse anti-FKBP-12 (1:100; Santa Cruz), rabbit anti-p70 S6K1 (1:1000; Cell Signaling, Cambridge, UK), monoclonal mouse anti-P-p70 S6K1-Thr389 (1:1000; Cell Signaling), rabbit anti P-AktSer473 (1:1000; Cell Signaling), rabbit anti-P-mTOR Ser2448 (1:1000; Cell Signaling), rabbit anti-4E-BP1 (1:1000; Cell Signaling), and Erk, JNK, p38 (Cell Signaling). The secondary antibodies in Odyssey Blocking Buffer (LI-COR) were incubated with the membrane for 1 h at RT and then analyzed by Odyssey CLx (LI-COR). The secondary antibodies were donkey anti-mouse Alexa 680 (1:15 000; Life Technologies) and goat anti-rabbit Alexa 800 (10000; LI-COR).

Cell viability assays

The Click-iT assay was completed by using the Click-iT EdU Alexa Fluor 647 Imaging Kit (Life Technologies) as described by the manufacturer, using 2-h incubation time for RPTECs.

Results

To investigate the effect of the mTOR inhibitor SIR on BKPyV replication, primary RPTECs were infected with BKPyV(Dun) for 2 h, before supernatants with unbound virions were removed and SIR was added at different final concentrations. As described previously in detail (38,39), BKPyV replication leads to a more than 100-fold increase in supernatant viral loads in the burst phase at 72 hpi in mock-treated cells, which was inhibited by SIR in a dose-dependent fashion (inhibitory concentration 90 at 4 ng/mL; Figure 1A). Immunofluorescence staining demonstrated that SIR treatment at 4 ng/mL was associated with a lower number of BKPyV-infected cells as shown for the LTag encoded in early viral gene region (EVGR) as well as for the cytoplasmic agnoprotein (Agno) or the nuclear major viral capsid protein Vp1 encoded in the late viral gene region (LVGR) (Figure 1B). As expected, SIR treatment also inhibited host cell proliferation to some extent, but normalization revealed that inhibitory effects were more pronounced for BKPyV replication, suggesting that virus infection rendered cells more susceptible to SIR inhibition (Figure 1C–E). The data indicated that SIR at clinically relevant concentrations was able to significantly inhibit BKPyV replication in primary human kidney cells.

To examine mTOR inhibition in more detail, SIR (4 ng/mL) was added at 2 hpi and intracellular BKPyV loads were compared with mock-treated BKPyV infection (Figure 2A). A significant inhibition of intracellular BKPyV loads was detected with approximately 10-fold lower intracellular viral genome loads at 36 hpi. This difference persisted at 48 hpi, even though some residual increase in BKPyV loads could be observed in SIR-treated cells, indicating that the replication block was significant, but not complete. To examine whether SIR inhibition was equally effective throughout the entire viral replication cycle, SIR (4 ng/mL) was added at different time points after infection and the respective supernatant BKPyV loads were measured at 72 hpi (Figure 2A). Inhibition of BKPyV replication was most pronounced when SIR was added in the first 2–12 hpi, and was no longer apparent from 24 hpi onwards (Figure 2B). The data suggest that early steps of the BKPyV replication cycle are particularly susceptible to SIR inhibition, which persisted during the time of EVGR expression up to 24 hpi, but not at later stages (i.e. encompassing the LTag-mediated viral genome replication and LVGR expression).

Comparing the time course of BKPyV protein expression in SIR- and mock-treated BKPyV-infected RPTECs by Western blotting revealed that SIR treatment at 2 hpi delayed and reduced expression of LTag, Vp1 capsid, and Agno over the 72 hpi (Figure 3A). One of the major downstream targets of mTOR is activation of the translational regulator p70-S6kinase by phosphorylation. As shown, BKPyV infection of RPTECs resulted in a pronounced activation of p70-S6kinase from 6 to 48 hpi as compared to mock-treated cells. Addition of SIR at 2 hpi almost completely blocked S6kinase phosphorylation over this entire time (Figure 3B).

BKPyV infection was also associated with an increase in phosphorylation of the serine-threonine kinase Akt, which is acting upstream of the mTOR-S6kinase-pathway (data not shown). Exposure of RPTECs to noninfectious BKPyV-VP1 virus-like particles also increased phosphorylation of Akt and, to a lesser extent, also of p70S6kinase involved in activation of mRNA translation machinery (Figure 3C). Further characterization revealed that BKPyV infection caused phosphorylation and activation of mTOR (Figure 3D), phosphorylation of and thereby inactivation of the translation inhibitory factor 4E-BP, another other downstream target of mTOR involved in translation regulation, as well as the kinases JNK and p38, but not of Erk1/2 compared to mock-treated RPTECs (Figure 3E). The data suggest that early steps during virion uptake and uncoating are sensed by the Akt-mTOR pathway and that the more pronounced strength and duration of the p70-S6kinase phosphorylation during BKPyV replication results from EVGR expression, in line with the discrete susceptibility of BKPyV replication to SIR inhibition up to 24–36 hpi.

To link the decrease in BKPyV replication more directly to mTOR inhibition, the effect of the mTOR kinase inhibitor torin-1 was investigated (44,45). Titration of increasing torin-1 concentrations was associated with reduction in BKPyV supernatant loads with >90% inhibition at 10 nM (Figure 4A). At this concentration, the p70S6kinase phosphorylation induced by BKPyV infection was also inhibited as observed for SIR (Figure 4B). Addition of torin-1 at different times after infection revealed a similar time dependence with significant inhibition of BKPyV supernatant loads up to 24 hpi, which disappeared thereafter (Figure 4C). The data support the view that mTOR activation is a central event in BKPyV infection, and that inhibition of mTOR by SIR or by torin-1 early in the viral life cycle results in significant decrease in BKPyV replication.

Since SIR inhibition of mTOR is mediated by binding to FKBP-12, it was of interest to examine the effects of FKBP-12 knockdown by siRNA. In the control RPTECs transfected with scrambled siRNA, SIR caused a dose-dependent decline in BKPyV LVGR proteins VP1 and Agno. This inhibitory effect of SIR was not seen in FKBP-12 knockdown RPTECs (Figure 5A). Similarly, BKPyV supernatant viral loads at 72 hpi declined in the siControl RPTECs, but remained unaffected in the siFKBP-12-knockdown cells (Figure 5B). The data indicate that SIR inhibits BKPyV replication through a pathway involving FKBP-12 binding and mTOR inhibition.

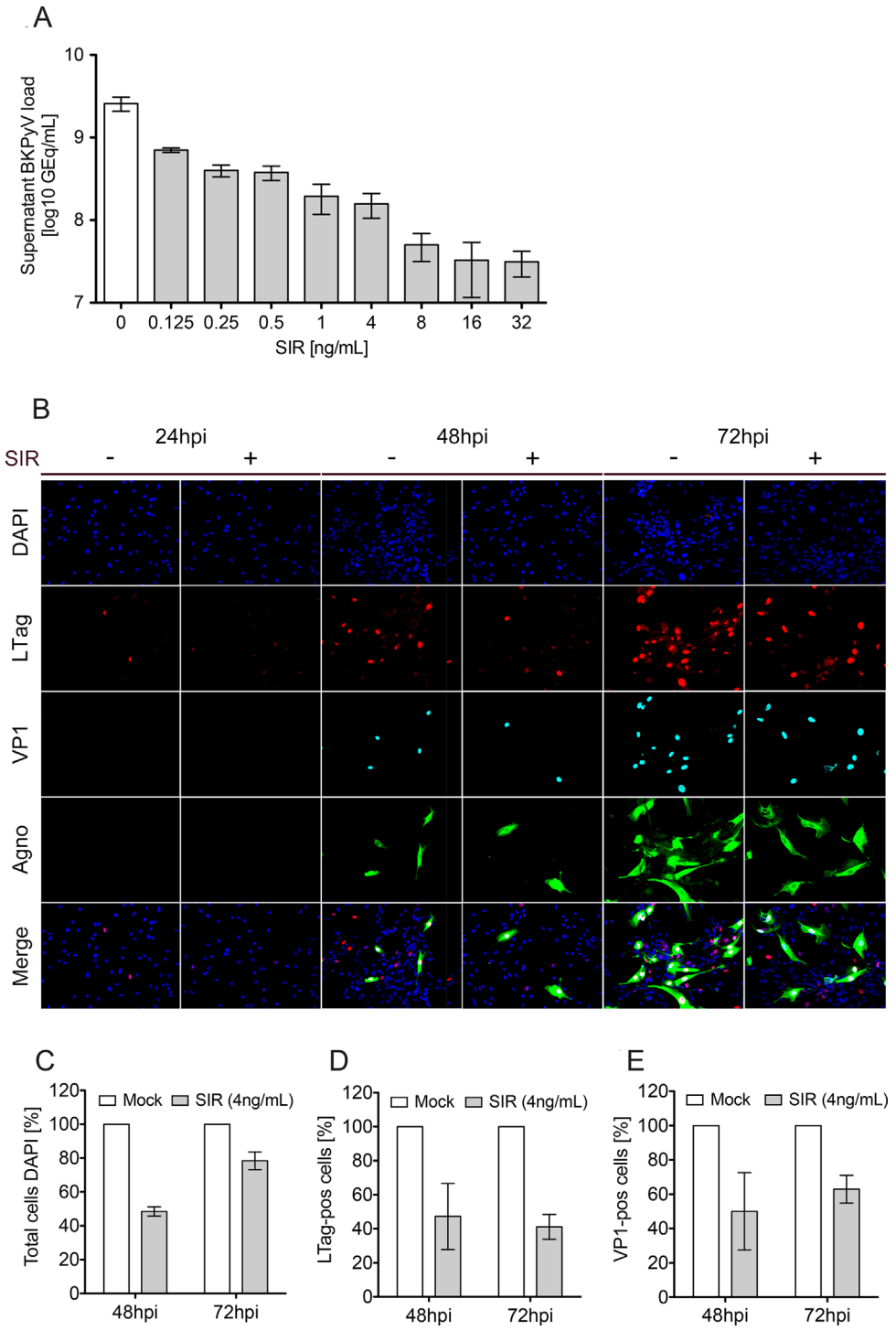

Figure 1: SIR inhibition of BKPyV replication. (A) SIR inhibition of supernatant BKPyV loads. RPTECs infected with BKPyV(*DUN*), mock (0), or SIR were added at 2 hpi at the indicated concentrations, and BKPyV load in supernatants was measured after 72 hpi. (B) SIR inhibition of BKPyV protein expression. RPTECS were infected with BKPyV(*DUN*) and at 2 hpi treated with SIR (4 ng/mL) or mock, and immunofluorescence staining was performed at 24, 48, and 72 hpi (red: large T-antigen, LTag; green: agnoprotein, Agno; cyan, capsid protein VP1; blue, DNA, and images were taken at 100× magnification). (C) Quantification of total cell count DAPI-positive cells; (D) LTag-positive cells; (E) VP1-positive cells. Cells were counted by Image J64 and normalized to the value of the mock-treated cells at the time points 48 and 72 hpi. BKPyV, BK polyomavirus; DAPI, 4′,6-diamidino-2-phenylindole; hpi, hours postinfection; LTag, large T-antigen; RPTECs, renal proximal tubule epithelial cells; SIR, sirolimus.

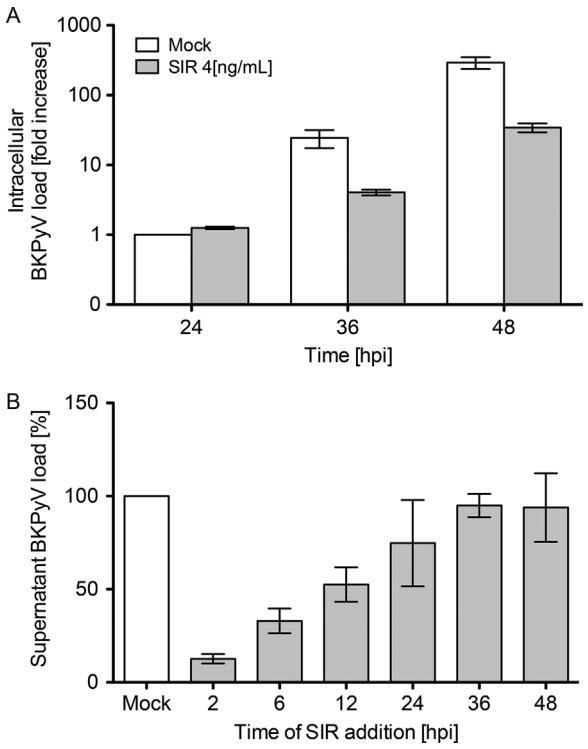

Figure 2: SIR inhibition of intracellular BKPyV load and time course. (A) SIR inhibits intracellular BKPyV replication. Infected RPTECs were treated with 4 ng/mL SIR at 2 hpi and lysed at 24, 36, and 48 hpi. Intracellular viral DNA was extracted and analyzed by qPCR for BKPyV load. The calculated values represented the GEq/150 000 cells and normalized to infected mock-treated cells at 24 hpi. (B) SIR inhibition is time dependent. Infected RPTECs were treated with 4 ng/mL SIR at the indicated time points postinfection. The supernatants were measured at 72 hpi and expressed as percent of mock treatment. BKPyV, BK polyomavirus; hpi, hours postinfection; qPCR, quantitative polymerase chain reaction; RPTECs, renal proximal tubule epithelial cells; SIR, sirolimus.

Since SIR and TAC are both known to bind to FKBP-12, it was of interest to investigate whether there was an additive or synergistic effect of the mTOR inhibitor SIR and the calcineurin inhibitor TAC with respect to BKPyV replication in RPTECs, as has been described for both of these drugs in immunosuppression of lymphocytes. Addition of TAC and SIR together at 2 hpi, however, reversed the inhibition of the supernatant viral loads observed for SIR alone (Figure 6A). TAC also reversed SIR inhibition of S6kinase phosphorylation in a dose-dependent manner (Figure 6B). The data indicate that TAC and SIR have opposing effects regarding BKPyV replication and mTOR inhibition in RPTECs. To investigate whether the SIR-antagonizing effect of TAC was mediated by FKBP-12, BKPyV replication was examined in siControl-transfected and siFKBP-12 transfected RPTECs in the presence and absence of TAC. The results demonstrated that supernatant BKPyV loads were increasing in response to increasing TAC concentration in RPTECs transfected with scrambled siRNA, whereas the BKPyV supernatant loads were high in the siFKBP-12 knockdown RPTECs and could not be increased further by TAC addition (Figure 6C). The data suggest that TAC activates and promotes BKPyV replication in RPTECs through a mechanism that operates similarly to knockdown of FKBP-12. Since SIR inhibition was only effective at a time before viral genome synthesis occurring at 24 hpi and depended on host cell DNA building blocks and the host cell DNA polymerase, we hypothesized that the TAC might affect overall host cell proliferation in a way opposite to SIR. Therefore, the effect of SIR and TAC was examined on host cell DNA synthesis rate using fluorescent labeling of newly synthesized DNA (Figure 7). The results demonstrate that SIR addition resulted in a decrease in new DNA synthesis in line with earlier data, whereas TAC caused an increase in host cell DNA synthesis.

To extend these observations to the calcineurin inhibitor CsA, BKPyV replication was compared in CsA- or in TAC-treated RPTECs. The respective drugs were added at 2 hpi and BKPyV loads in the supernatants were measured (Figure 8). As shown, TAC treatment resulted in an accelerated BKPyV replication about 1 day earlier than mock-treated cells, whereas CsA treatment resulted in a lower BKPyV load. The data indicate that the calcineurin inhibitors had opposing net effects on BKPyV replication in RPTECs, despite similar effects inhibitory effects on T cell activation.

Discussion

In this study, we investigated the direct effects of immunosuppressive drugs on BKPyV replication in primary human renal proximal tubular epithelial cells, the viral target in BKPyV-associated nephropathy. Our results demonstrate that there are fundamental differences between SIR and TAC: At clinically relevant concentrations, SIR inhibits BKPyV replication, while the opposite is true for TAC. TAC activation and SIR inhibition of BKPyV replication require, and compete for, FKBP-12, a small host cell protein known to bind to either drug. Some details of our results are worth considering for a balanced interpretation of the study.

SIR concentrations of ≈4 ng/mL inhibit BKPyV progeny release at 72 hpi and correlate with a decline in viral proteins. As expected for inhibiting mTOR kinase activity, SIR addition prevents the activation of relevant downstream targets such as p70-S6kinase and 4E-BP (46,47). S6kinase and 4E-BP phosphorylation is rapidly blocked within less than 2 h of drug addition and lasts throughout the entire 72 h of the BKPyV replication cycle. However, BKPyV replication is not equally susceptible throughout this time. In fact, SIR inhibition is most pronounced when added during the early phase of BKPyV replication up to 24 hpi, while disappearing almost completely during the late phase. The loss of susceptibility to SIR inhibition is not due to a recovery of mTOR activity, as indicated by the persisting block of p70-S6kinase- and

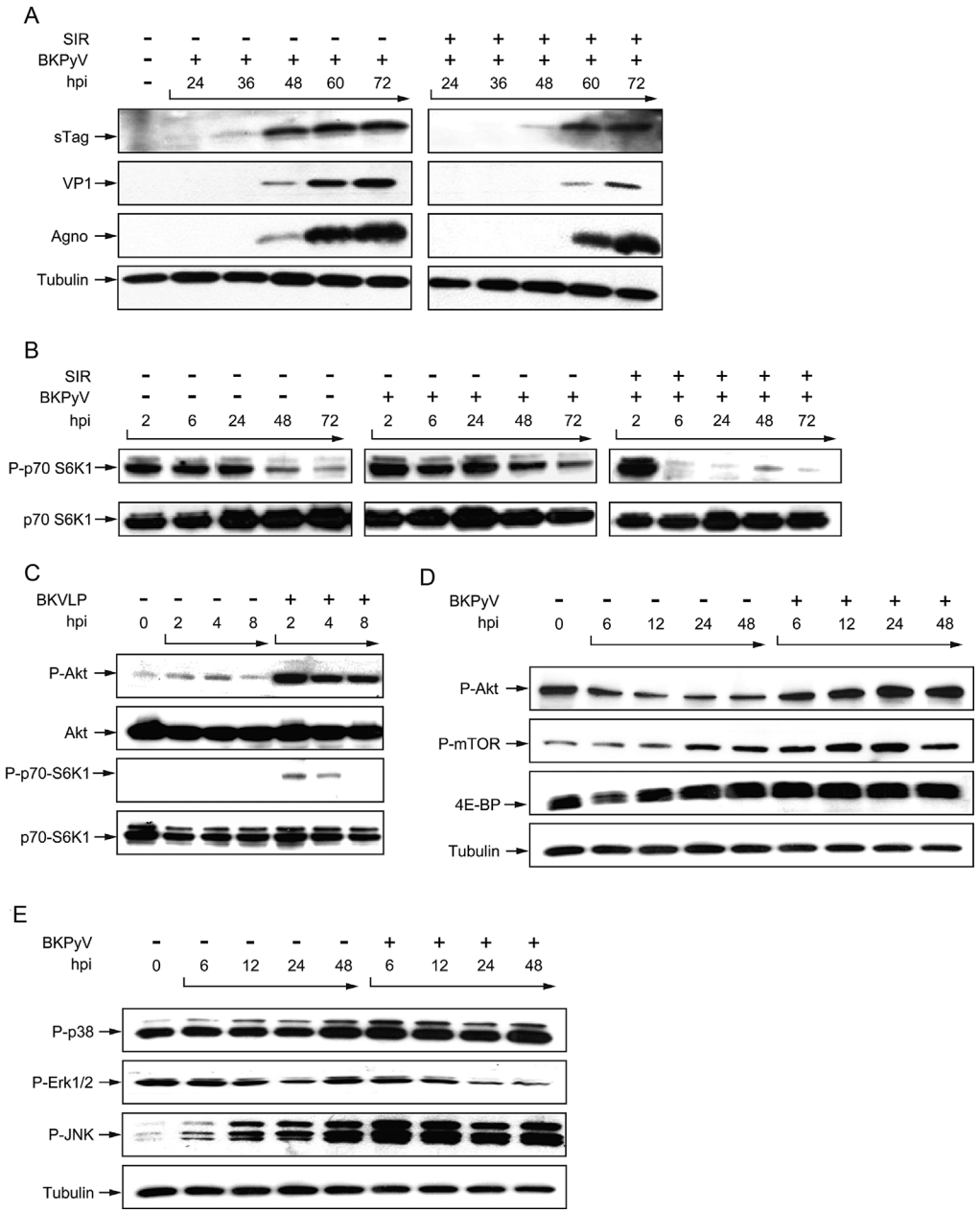

Figure 3: SIR treatment reduces BKPyV protein expression and blocks mTOR pathway activated by BKPyV infection. Cell lysates were prepared at the different times postinfection in the presence and absence of SIR (4 ng/mL) as indicated and analyzed with Western blotting for the indicated viral and host cell proteins. (A) SIR delays and reduces BKPyV protein expression. The EVGR protein small T-antigen (sTag) and LVGR proteins VP1 and Agno are analyzed by Western blot as indicated, and tubulin was used as a loading control. (B) BKPyV infection activates the mTOR-p70S6Kinase pathway. Cell lysates were prepared at the indicated times after BKPyV infection in the presence or absence of SIR (4 ng/mL) and analyzed by Western blotting on 10% SDS-PAGE for 70S6kinase phosphorylation (P-p70SK1) and total p70S6Kinase (p70S6K1). (C) BKPyV virus-like particles (BKVLP) activate Akt-mTOR pathway. RPTECs were starved for 48 h and then exposed to BKVLPs (hemaglutination titer of 1:3200) or mock treatment before preparing cell lysates for Western blot analysis on 10% Tris-Tricine gel for the indicated targets P-Akt, total Akt, P-p70 S6K1, and total p70 S6K1. (D) BKPyV infection activates the Akt-mTOR pathway. RPTECs were starved for 48 h and then exposed BKPyV(*DUN*) virions before preparing cell lysates for Western blot analysis for the indicated targets P-Akt, P-mTOR, 4E-BP, and tubulin. (E) BKPyV infection activates p38 and JNK kinase pathway. RPTECs were starved for 48 h and then exposed BKPyV(*DUN*) virions before preparing cell lysates at the indicated time points for Western blot analysis for the indicated targets P-p38, P-Erk1/2, P-JNK, and tubulin. Agno, agnoprotein; BKPyV, BK polyomavirus; 4E-BP, translation inhibitory factor 4E binding protein; EVGR, early viral gene region; hpi, hours postinfection; LVGR, late viral gene region; mTOR, mammalian target of rapamycin; P-Akt, phosphorylated serine-threonine kinase-Akt; SDS-PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; SIR, sirolimus.

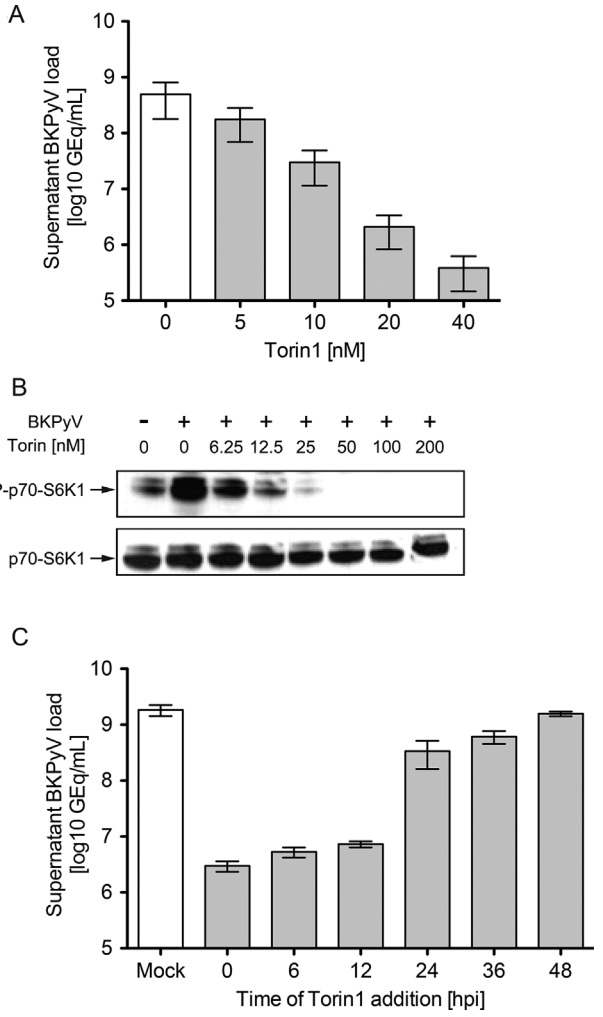

Figure 4: Torin1 inhibits BKPyV replication in RPTECs. (A) Dose-dependent inhibition of supernatant BKPyV loads. RPTECs were infected with BKPyV(*DUN*) for 2 h, and treated with the indicated final concentrations of Torin1. At 72 hpi, the BKPyV load in culture supernatants was determined by qPCR. (B) Dose-dependent inhibition of p70S6kinase phosphorylation. RPTECs were infected with BKPyV(*DUN*) for 2 h, and treated with the indicated final concentrations of Torin1. Cell lysates were prepared at 6 hpi and analyzed by Western blotting for P-p70-S6K1 and total p70 S6K1. (C) Time-dependent inhibition of BKPyV replication. RPTECs were infected with BKPyV(*DUN*) for 2 h, and treated at the indicated time points postinfection with 100 nM of Torin1. At 2 hpi, 100 nM Torin1 was added. At 72 hpi, the BKPyV load in culture supernatants was determined by qPCR. BKPyV, BK polyomavirus; hpi, hours postinfection; p70-S6K1, total p70S6Kinase; P-p70-S6K1, phosphorylated S6-kinase of 70kD; qPCR, quantitative polymerase chain reaction; RPTECs, renal proximal tubule epithelial cells.

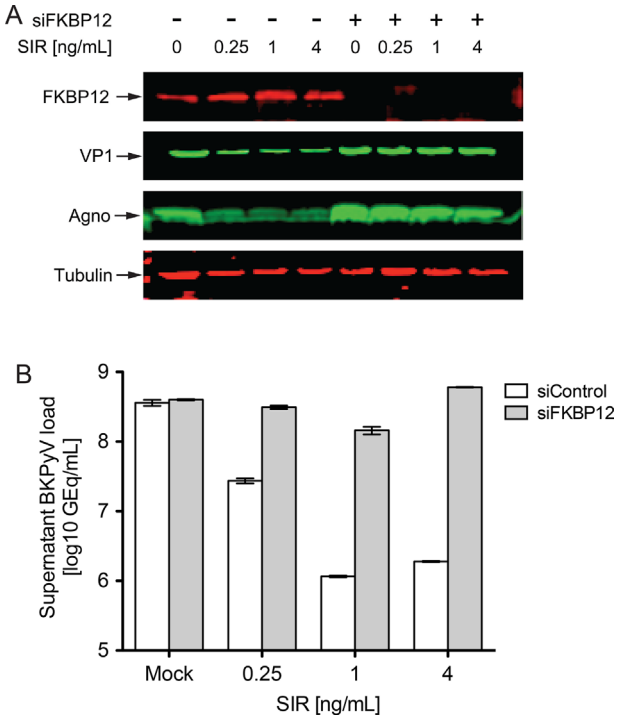

Figure 5: Comparing SIR treatment on BKPyV replication in RPTECs after FKBP-12 knockdown. (A) RPTECs were seeded into a T25 flask and FKBP-12 was transfected with siRNA tFKBP-12 targeting siRNA (+) or scrambled control siRNA (–) as described in Materials and Methods. On the next day, the respective RPTECs were seeded ($2.5^{*}10^{5}$ cells/well; six-well plate) and left to adhere overnight. The cells were infected with BKPyV and treated at 2 hpi with the indicated final concentrations of SIR. At 48 hpi, cell lysates were prepared and analyzed by a 15% Tris-Tricine SDS-PAGE and Western blotting for FKBP-12, BKPyV VP1, Agno, and tubulin. (B) RPTECs transfected with siRNA-FKBP-12 and siRNA control were infected with BKPyV and treated with SIR (4 ng/mL) at 2 hpi. At 72 hpi, the BKPyV load in culture supernatants was determined by qPCR. Agno, agnoprotein; BKPyV, BK polyomavirus; FKBP, FK binding protein; hpi, hours postinfection; qPCR, quantitative polymerase chain reaction; RPTECs, renal proximal tubule epithelial cells; SDS-PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; SIR, sirolimus; VP1, viral capsid protein 1.

4E-BP-phosphorylation. Importantly, the inhibition of mTORC1 by torin-1, a FKBP-12 independent inhibitor unrelated to the mTORi drug class of SIR (45,48), shows a similar time-dependent inhibition profile in line with the presence of an early mTOR-dependent and late mTOR-independent phase of BKPyV replication.

Comparing the response of primary human renal tubular epithelial cells to noninfectious BKPyV virus-like particles and infectious virions revealed that the Akt-mTOR-S6kinase pathway is activated early and independent of the infectious potential of the particles, indicating that the cells sense receptor engagement and uptake. The stronger and longer-lasting mTOR stimulation elicited by infectious virions argues for a contributory role of EVGR expression known to start after 6–12 hpi (38,39). The Akt-mTOR-S6kinase activation and the time-dependent mTOR inhibition suggest that host cell activation is important for uptake

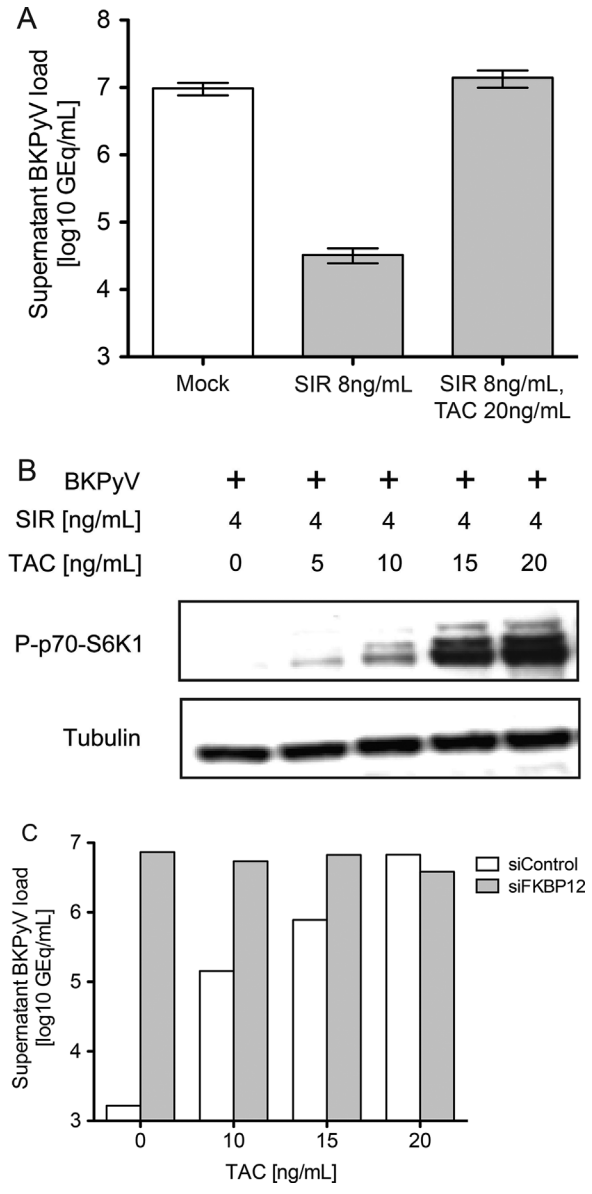

Figure 6: TAC reverses SIR inhibition of BKPyV replication in RPTECs. (A) BKPyV supernatant loads. RPTECs were infected with BKPyV for 2 h, and treated with the indicated final concentrations of SIR and TAC as described in Materials and Methods. At 72 hpi, the BKPyV load in culture supernatants was determined by qPCR. (B) p70S6 Kinase phosphorylation. RPTECs were infected with BKPyV for 2 h, and treated with the indicated final concentrations of SIR and TAC as described in Materials and Methods. At 6 hpi, cell lysates were analyzed by Western blotting for P-p70 S6K1 and p70 S6K1. (C) Effect of TAC on BKPyV replication in RPTECs after FKBP-12 knockdown. RPTECs transfected with siRNA FKBP-12 or scrambled control were infected with BKPyV for 2 h, and then treated with the indicated final concentrations of TAC. At 72 hpi, the BKPyV load in culture supernatants was determined by qPCR. BKPyV, BK polyomavirus; FKBP, FK binding protein; hpi, hours postinfection; P-p70 S6K1, phosphorylated S6-kinase of 70kD; qPCR, quantitative polymerase chain reaction; RPTECs, renal proximal tubule epithelial cells; SIR, sirolimus; TAC, tacrolimus.

and efficient EVGR expression. This is further enhanced by BKPyV EVGR expression such as LTag. The potential virological implications are that translation of the LVGR-mRNAs encoding the viral capsid proteins can occur largely independent of cap-dependent translation and may thereby proceed through the final viral burst phase despite host cell exhaustion, a state that is normally sensed and restricted by mTOR (49). Conversely, the inhibitory effects of SIR are most pronounced when the drug is present prior to infection as in a prophylactic situation, while its effects on ongoing BKPyV replication may only emerge with new rounds of host cell infection (44).

SIR inhibition of BKPyV replication has been previously examined in HK2 cells, a transformed human kidney cancer cell line (50). However, the inhibitory SIR concentrations required were at least one order of magnitude higher than the ones observed here for primary RPTECs, and hence in the clinically toxic range (51–53). This suggests that the susceptibility to BKPyV might be enhanced by the transformed phenotype of cancer host cells, which often involve altered signal transduction and metabolic pathways (54,55). For our study, we used the well-characterized BKPyV(Dun) strain, which is derived from the original Gardner strain (56), and replicates similarly to natural variants found in KT patients (16). Also, different donor sources of RPTECs were used with indistinguishable results, suggesting that our results would apply to most KT recipients.

As shown, SIR-mediated inhibition of BKPyV replication is dependent on FKBP-12, the adaptor protein of SIR required for mTOR inhibition. FKBP-12 is a 12-kDa cytoplasmic protein originally identified as one of many cellular proteins binding to ''FK506,'' the former experimental name of the drug now called TAC. Remarkably, increasing concentrations of TAC reverse SIR inhibition of both BKPyV replication and the mTOR-S6kinase pathway, suggesting that both drugs compete for binding for FKBP-12 as a common target. Knockdown of FKBP-12 by siRNAs abrogates SIR inhibition of BKPyV replication, as evidenced by higher supernatant viral loads and stronger viral protein expression compared to mock-treated scrambled siRNA controls. FKBP-12 knockdown also abrogated the dose-dependent activation of BKPyV replication by TAC, but not by preventing BKPyV replication, but by replicating already at a high level in the absence of TAC. Thus, BKPyV replication in FKBP-12 knockdown cells is similar to one in control cells treated with higher concentrations of TAC around 10–20 ng/mL. These data suggest that FKBP-12 is centrally involved in BKPyV replication by having a naturally negative ''hand brake'' effect on BKPyV replication that can be turned off by TAC binding. The effect of TAC on BKPyV replication is unlikely to result from its actions as calcineurin inhibitor, since the CNI CsA exerted an inhibitory effect on BKPyV replications, as has been reported in some detail by others (57–59), at concentrations that are active in T cells (60). While the precise mechanisms underlying TAC activation of BKPyV replication through FKBP-12 require

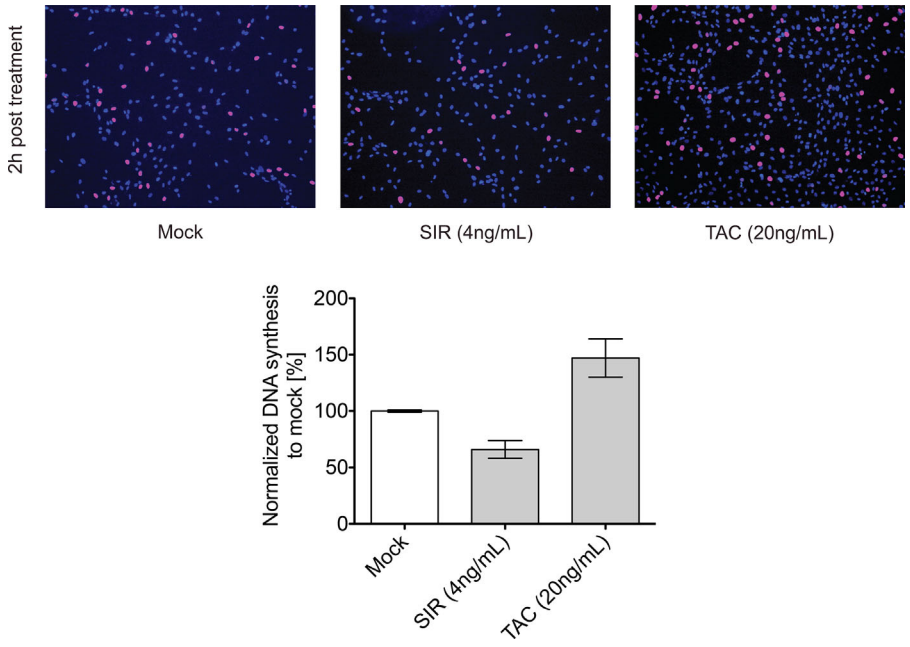

Figure 7: The effect on SIR and TAC on the DNA synthesis in RPTECs. RPTECs were seeded onto coverslips overnight, and mock-treated, or treated with 4 ng/mL SIR, or 20 ng/mL TAC for 22 h. EdU labeling was performed for 2 h and then the cells were fixed and analyzed with immunofluorescence. Cellular DNA (blue) and the newly labeled cellular DNA (pink). Image J64 was used to quantify and normalize the Hoechst-positive and EdU-positive cells. RPTECs, renal proximal tubule epithelial cells; SIR, sirolimus; TAC, tacrolimus.

further study, our data indicate that TAC addition resulted in an activation host cell DNA synthesis, while the opposite was seen for SIR. Accordingly, TAC and SIR appear to influence the overall renal tubular epithelial cell milieu by rendering these cells more or less conducive to BKPyV replication, respectively (Figure 9). The direct effects of mycophenolate have been examined in monkey Vero-E6 cells, and were reported to inhibit BKPyV replication (61), but investigating this mycophenolate effect in human RPTECs may be warranted.

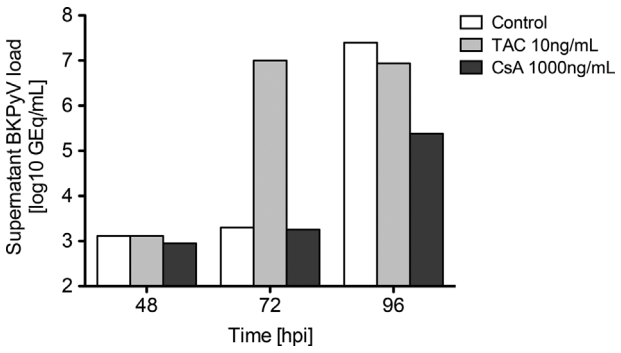

Figure 8: TAC stimulates and CsA inhibits BKPyV replication in RPTECs. RPTECs were infected with BKPyV for 2 h, and treated with the indicated final concentrations of TAC and CsA as described in Materials and Methods. At 72 hpi, the BKPyV load in culture supernatants was determined by qPCR. BKPyV, BK polyomavirus; CsA, cyclosporine A; hpi, hours postinfection; qPCR, quantitative polymerase chain reaction; RPTECs, renal proximal tubule epithelial cells; TAC, tacrolimus.

What are the potential clinical implications of our observations, and specifically for KT? The increased risk of BKPyV complications in TAC-containing regimens compared to mTORi- or CsA-containing regimens (21,22,25) could reflect the higher immunosuppressive potency of TAC regimens or differences in BKPyV-promoting or –inhibiting properties. In fact, both hypotheses are not mutually exclusive, and may actually play out in different times and clinical settings posttransplant. Although individually adapted to the immunologic risk of a given donor–recipient pair, the intensity of immunosuppression is usually highest at and shortly after transplantation. As dosing is lowered after 3 months posttransplant, however, drug-specific differences may become apparent: BKPyV-activating effects of TAC may increase the risk compared to drugs with BKPyV-inhibitory effects such as CsA, mTOR inhibitors, at an otherwise appropriate maintenance immunosuppression. There are only a few prospective randomized studies of sufficiently large sample size that provide some information on immunologic potency as well as BKPyV replication (9,24,33,34). The DIRECT study of more than 600 *de novo* kidney transplant patients reported non-inferiority of the standard-dosed TAC-mycophenolate arm and the C2-monitored CsA-mycophenolate arm for biopsy-proven acute rejection at 6 months posttransplant, or for a

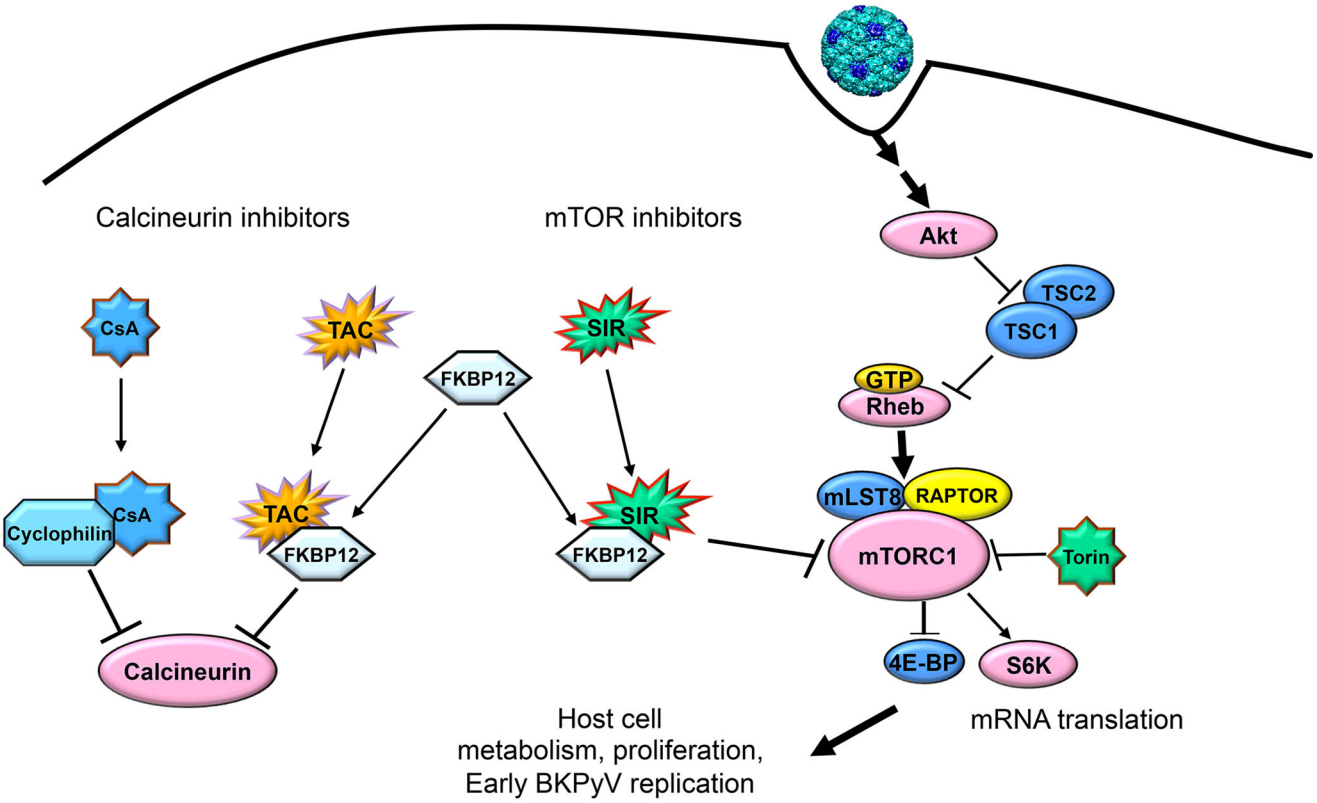

Figure 9: Effects of calcineurin inhibitors and mTOR inhibitors on BKPyV replication. Akt, plasma membrane located, inositol-activated serine-threonine kinase; BKPyV, BK polyomavirus; CsA, cyclosporine A; FKBP, FK binding protein; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; SIR, sirolimus; S6K, S6 kinase; TAC, tacrolimus; TSC, tuberous sclerosis factor; 4E-BP, translation inhibitor 4E binding protein.

composite end point including rejection, graft loss, or death (33). This immunologic noninferiority at 6 months posttransplant was contrasted by an increased rate BKPyV viremia in the TAC arm with significantly higher BKPyV loads as compared to the CsA arm, and only emerged after 3 months posttransplant (24). In the A2309 study comparing the mTORi everolimus and reduced-exposure CsA with standard-exposure CsA-mycophenolate, immunologic potency was noninferior according to treated biopsy-proven rejection or a composite end point including also graft loss and death at 12 months posttransplant, but BKPyV replication was significantly less frequent in the everolimus arm (34). These data support the view that off-target effects of immunosuppressive drugs on BKPyV replication in the renal allograft may play out at intermediate levels of immunosuppression that are able to control alloimmune responses. Thus, our study provides virological rationales for clinical trials that may attempt to harness the direct BKPyV-activating or –inhibiting activities of immunosuppressive drugs in order to better control BKPyV replication in KT. This knowledge could be important for prevention and treatment of BKPyV complications in the absence of, or as an adjunct to, BKPyV-specific antiviral therapies that are clearly needed.

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Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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